Korean J. of Pharmacology
Vol. 24, No. 2, pp. 233-240, 1988

A Simple, Sensitive, and Specific HPLC Analysis of Tissue Polyamines using FNBT Derivatization: Its Application on the Study of Polyamine Metabolism in Regenerating Rat Liver

Sang-Hyun Choi, Hyung-Gun Kim, Hong-Ik Park and Boe-Gwun Chun

Department of Pharmacology, Korea University College of Medicine

ABSTRACT

A simple and selective isocratic HPLC method for the analysis of tissue polyamine contents is described and applied on the study of the changes of the hepatic polyamine contents after partial hepatectomy in male rats. The hepatic polyamines are extracted with 0.4 M perchloric acid containing 2 mM disodium EDTA, and then the extract is redissolved in 100 ul of 1 M sodium carbonate and incubated with 300 ul of FNBT-dimethylsulfoxide (1: 100) mixture. The N-2'-nitro-4'-trifluoromethylphenyl drivatives of polyamines are separated through a ERC-ODS column in an isocratic mode with an acetonitrile-water (80:20) mobile phase within 20 min. per a sample, while monitoring the effluent at 242 nm.

This improved method which could detect subnanogram of each polyamines is highly specific and reproducible as evidenced by the application of it on the study of the changes of polyamine contents in the regenerating rat liver after partial hepatectomy.

Key Words: Polyamine, Putrescine, Spermidine, Spermine, Regenerating Liver, HPLC analysis

Abbreviations: FNBT; 4-fluoro-3-nitrobenzotrifluoride, NTP-polyamine (N-2'-nitro-4'-trifluoromethylphenyl polyamine, MGBG; methylglyoxal bis(guanylhydrazone) dihydrochloride, AdoMet; S-adenosyl-L-methionine, MTA; 5'-deoxy-5'-methylthioadenosine, ODC; ornithine decarboxylase, HPLC; high performance liquid chromatography

INTRODUCTION

During the last thirty years, great interest has been increasing in the naturally occuring putrescine (1,4-diaminobutane), spermidine, and spermine, which are present in most tissues and have been shown to play vital roles in cell growth and differentiation (Williams-Ashman *et al.*, 1979; Pegg and McCann, 1982; Tabor and Tabor, 1984)

The biosynthesis pathways for the diamine putrescine and the polyamine spermidine and spermine are well established (scheme in Fig. 1), and the two enzymes involved in the specific steps: namely, ornithine decarboxylase (ODC) and adenosylmethionine (AdoMet) decarboxylase are of particular interest, because of their extraordinary high turnover rate and their rapid response to a variety of hormonal and other stimuli (Tabor and Tabor, 1976; 1984).

By the use of ODC inhibitors, Pösö and Jänne (1976), Pösö and Pegg (1982), and Luk (1986) have shown that ODC may be essential for liver regeneration. However, Harik *et al.* demonstrated that the inhibition of polyamine synthesis might not result in decreases of DNA and RNA synthesis. And it was shown that spermidine and 5'-deoxy-5'-methylthioadenosine (MTA) exercise a negative control in vivo on the activities of AdoMet decarboxylase and spermidine/spermine synthases, respectively (Mamont *et al.*, 1981; Kamatani *et al.*, 1980).

Therefore, the interrelated metabolic pathway of polyamines suggests that the simultaneous, selective, and quantitative determination of putres-

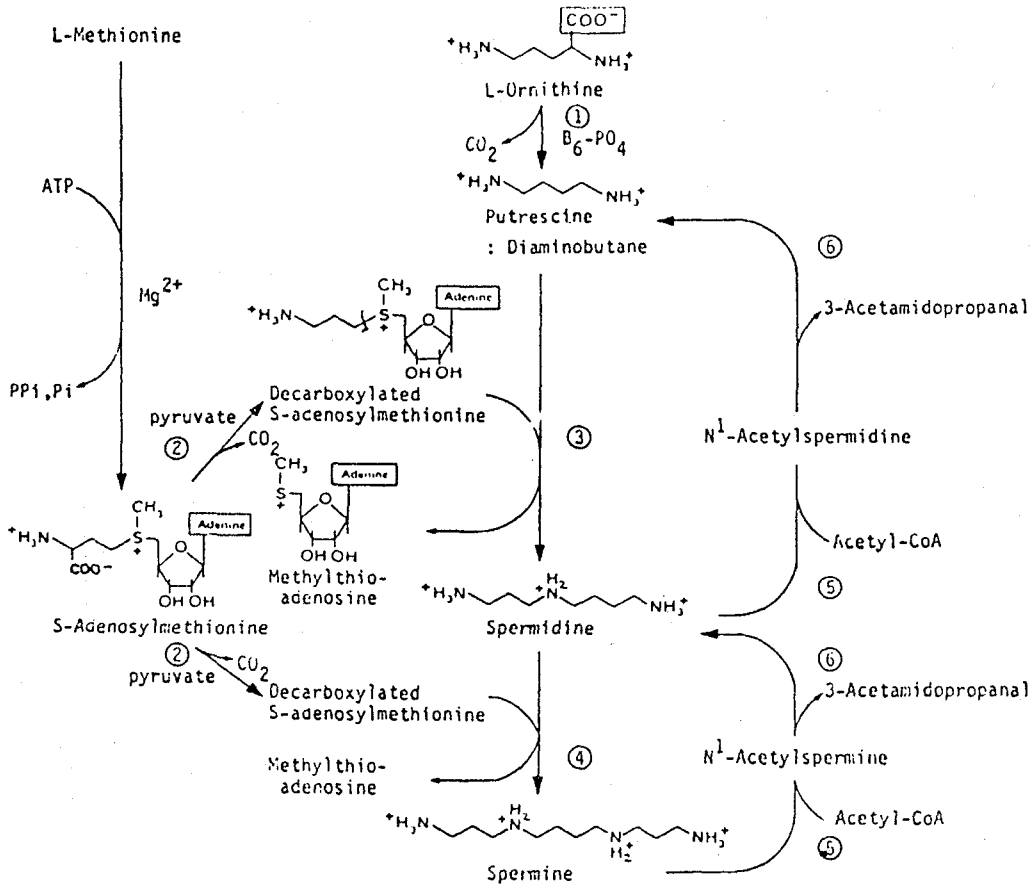

Fig. 1. Pathway for biosynthesis and interconversion of polyamines. The enzymes involved are: (1) ornithine decarboxylase, (2) S-adenosylmethionine decarboxylase, (3) spermidine synthase, (4) spermine synthase, (5) spermidine/spermine N^1-acetyltransferase, (6) polyamine oxidase.

cine, spermidine, and spermine should greatly enhance the study for the biological roles of them.

As appears from previous reviews (Bachrach, 1978; Seiler, 1986), all chemical methods for polyamine analysis require at least two steps: separation from all other amino groups and derivatization to allow their sensitive detection. Because these derivatizations have been not specific for the polyamines, the specificity of the chemical methods is exclusively based on time-consuming and uneconomical separation procedures.

In the present paper, the simple, selective, and sensitive HPLC method, that was originally described by Spragg and Hutchings (1983) but have never applied in biological smaples is employed on the study of the changes of polyamine contents in regenerating rat liver after partial hepatectomy.

MATERIALS AND METHODS

Materials

The hydrochlorides of putrescine, spermidine, spermine and histidine were purchased from Sigma. 1,8-Diaminooctane, 4-fluoro-3-nitrobenzotrifluoride, methylglyoxal bis(guanylhydrazone) dihydrochloride, dimethyl sulfoxide and 2-methylbutane were from Aldrich. Acetonitrile was a chromatographic grade preparation of Merck.

Other chemicals were analytical grade. Male

Sprague-Dawley rats (Weighing 150 to 180 g) were obtained from Kor. Exp. Animal Lab.

Treatments of animals

Male Sprague-Dawley rats were kept 2 to a cage, and allowed to acclimate to a 12 hr-light (7 AM to 7 PM) and 12 hr-dark cycle for one wk before being studied. The rats were subjected to partial hepatectomy of 70.4±1.99% under light ether anesthesia between 9 and 12 AM (Higgins and Anderson, 1931), and the remnant liver weight was estimated 29.7±1.45% of total liver weight. Sham-operated animals underwent a similar laparotomy procedure.

The MGBG-treated rats were given a subcutaneous injection of 40 mg/kg MGBG in 0.85% NaCl solution 2 hr before hepatectomy and every 10 hr for 50 hr after the first injection.

Extraction and derivatization of polyamines

Excised livers obtained after decapitation were homogenized with a tapered teflon homogenizer in 5 vol. of cold 0.4M perchloric acid/2mM disodium EDTA containing diaminooctane (100 μg) as an internal standard. Homogenation and further extraction steps were done at 0-6℃. One ml of the homogenate was spun at 15,000 x g for 15 min and 100 ul of the supernatant was transferred to an 1.5 ml microfuge tube and then evaporated to dryness with streams of nitrogen gas at room temperature. For derivatization with FNBT (Spragg and Hutchings, 1983), the dry residue obtained was redissolved in 100 μl of 1M sodium carbonate.

This solution was treated with 300 μl of FNBT reagent (10 μl FNBT/ml dimethyl sulfoxide), and after mixing, the reaction was allowed to proceed at 60℃ for 20 min. At the end of this time, 40 μl of 1M histidine in 1M sodium carbonate was added to the reaction mixture and the incubation continued for a further 5 min.

After cooling the mixture, the N-2'-nitro-4'-trifluoromethylphenyl polyamine (NTP-polyamine) derivatives were extracted twice with 2 ml of 2-methylbutane. After centrifuge at 1500 x g for 5 min, the organic phase was evaporated to dryness in a conical centrifuge tube with streams of nitrogen gas, and the residue was reconstituted with 1 ml of HPLC-grade methanol; 20 μl of the methanol solution was applied to the HPLC-analysis.

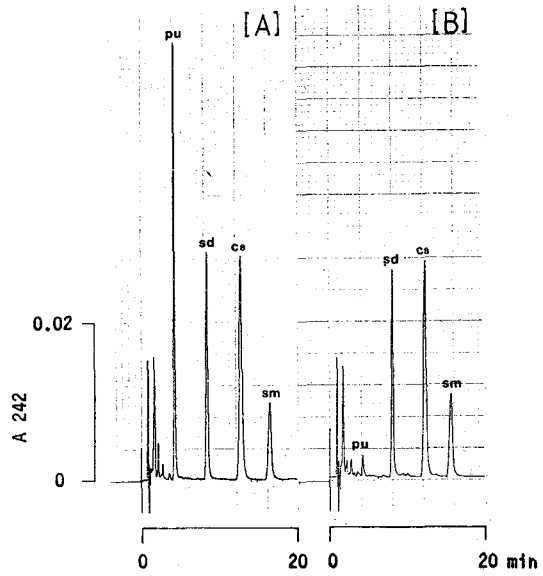

Fig. 2. Isocratic HPLC chromatograms of
(A) a mixture of polyamines containing 50 ng of each authentic standard and
(B) a sample equivalent to 250 μg of rat liver with 50 ng of diaminooctane.

Abbreviations-
pU:putrescine, sd: spermidine
sm: Spermine, C8: diaminooctane

HPLC apparatuses and separation

The HPLC system was composed of a Knauer HPLC pump (W. Germany), a Rheodyne-7125 sample injector, a Knauer variable UV/VIS monitor, a ERC ODS-1161 column (3 um, 6×100 mm; Japan), and a Linear chart recorder.

The 20 μl of the methanol solution obtained above was applied on a reversed-phase HPLC system equipped with a short ODS column, and then the separation of NTP-polyamines was completely accomplished by an isocratic elution of acetonitrile-water (80:20) mobile phase at the rate of 1.2 ml/min within 20 min (Fig. 2). The absorbance of effluent at 242 nm was monitored on a 2-channel chart recorder. The all calibration curves were consistently linear over a range of 50 picomole to 10 nanomole with less than 5% variation between identical samples (Fig. 3), and the detection limit was somewhat less than 10 picomole on column with a S/N ratio of 5:1.

The amount of each compound per gm of wet liver was first estimated directly from the calibra-

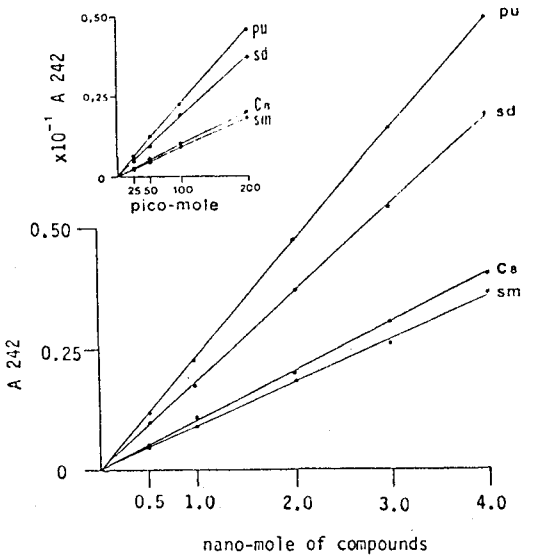

Fig. 3. Calibration curves of authentic polyamines.

tion curve based on the peak heights, and the values were corrected according to the recovery factor of each authentic standard. The recovery of each polyamine was calculated as follows; each compound of known amount equivalent to the amount of the amine in the normal rat liver was added to the initial homogenate of perchloric acid, which served as the internal standard; and then the absorbance of each standard was corrected by subtracting the absorbance due to the endogenous origin of the perchloric acid homogenate without added standard.

RESULTS AND DISCUSSION

Increased polyamine synthesis and accumulation occur in all growth and proliferation processes studied to date, including embryonic development, tissue regeneration, cellular hypertrophy, oncogenesis, and cell cycle transverse in culture (Williams-Ashman *et al.*, 1979; Russel and Haddox, 1979; Pegg and McCann, 1982; Womble and Russell, 1983; Tabor and Tabor, 1984). Thus it appears that a demand has been greatly increasing for more simple and rapid or more complete and precise assay procedures for the polyamines and their derivatives. Numerous methods for determination of polyamines have been described (Seiler, 1986), including the usage of HPLC.

Polyamines have not any structural feature that would allow their sensitive detection without derivatization. And the concentration of them in tissues, body fluids and urine is in the micromoles per litre range, comparable to that of many other biogenic amines and amino acids, with which they share the amino groups as the most conspicuous structural feature. As appears from previous papers (Bachrach, 1978; Seiler, 1986), therefore, most HPLC methods for polyamine analysis require either multi-buffer elution with precolumn or postcolumn derivatization.

And all chemical methods of polyamine analysis require at least two steps: laborous and time-consuming separation from other amine compounds of biological samples and derivatization with light absorbing or fluorogenic agents such as benzoyl chloride, p-toluenesulphonyl chloride, 4-fluoro-3-nitrobenzotrifluoride, dansyl chloride, o-phthalaldehyde, fluorescamine, and so forth.

Recently, Spragg and Hutchings (1983) reported an isocratic HPLC method for polyamine analysis based upon the formation of NTP-polyamine derivatives using FNBT, which Vessman and Stromberg (1977) had first used as a derivatizing agent of tranexamic acid. But any application of the isocratic method for polyamine analysis in biological samples has not been carried out. In this paper, therefore, the possible use of the method for the polyamine determination of biological samples was investigated on the study of the changes of hepatic polyamine contents in regenerating rat liver after partial hepatectomy described by Higgins and Anderson (1931).

Compared with the previously reported HPLC methods for polyamine analysis using derivatization techiques, the method presented here using FNBT as a derivatizing agent, in addition to general advantages of precolumn derivatization method (Krstulovic and Brown, 1982; Seiler, 1983), has the advantages of the stability of the derivatives, the mostly desirable resolution efficiency as illustrated in capacity factors (Table 1), and not forming side reaction products (Fig. 2). And the isocratic HPLC method presented here has the picomolar sensitivity (Table 1; Fig. 3), comparable to those of other methods (Samejima *et al.*, 1976; Whitemore and Slotkin, 1985; Bachrach and Plesser, 1986), eliminates the needs for the laborous time-consuming sample preparation (Seiler, 1983; Endo, 1983; Whitemore and Slotkin, 1985) and expensive multi-pump HPLC system.

The isocratic reversed-phase HPLC method for the simple, specific, sensitive, and time-saving

Table 1. Isocratic HPLC parameters of the NTP-polyamine derivatives

Polyamine	capacity factor (k')	sensitivity (pmole)	recovery rate (%)
putrescine(pu)	4.12	1.55	77.24
spermidine(sd)	9.25	1.97	84.29
diaminooctane(C_8)	14.37	3.47	72.10
spermine(sm)	18.75	3.59	88.46

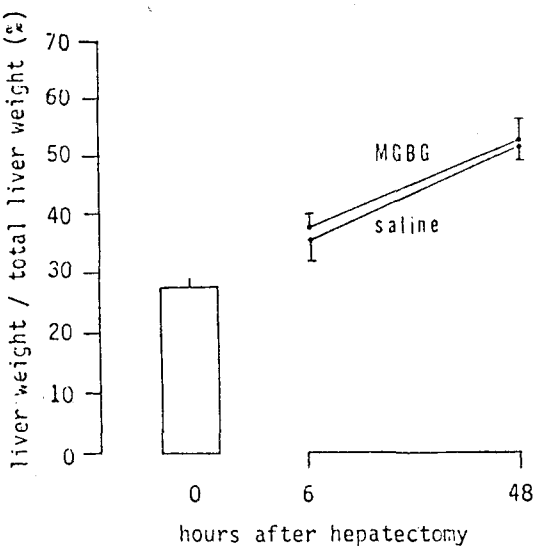

Fig. 4. Influence of MGBG on the weight recovery of the regenerating liver after partial hepatectomy.
Data indicate the mean±standard error of 4 to 6 samples.

analysis of polyamine; putrescine, spermidine, and spermine could be evaluated to the study of the polyamine metabolism in regenerating rat liver after partial hepatctomy. In this study, the mortality of parital hepatectomy within the first 48 hr was 7.9%, comparable to other studies (Higgins and Anderson, 1931; Luk, 1986). And hepatectomized rats treated with MGBG, a reversible inhibitor of AdoMet-decarboxylase (Pegg, 1983; and Seppanen *et al.*, 1983), did not show any statistical difference in the surgical mortality. The control group treated with saline and the experimental group treated with MGBG had rapid increase in liver weight, increasing from 29.7±2.45% of prehepatectomy liver weight to 52.1% and 53.3%, respectively, at 48 hr (Fig. 4), similar to other papers (Zieve *et al.*, 1985; Luk, 1986).

After partial hepatectomy, the putrescine content of rat liver (control value; 158.7±14.1 nanomole/g wet liver) was rapidly increased to 660.2±32.2 nanomole/g wet liver at 6 hr and fell down to the normal value at 48 hr, and the normal spermine content (control value; 876.6±42.1 nanomole/g wet liver) showed the initial slight increase at 6 hr followed by declining to the normal value at 48 hr (Fig. 5). But the spermidine content of prehepatectomy control group (829.7± 36.4 nanomole/g wet liver) exhibited an initial rapid increase (1316.1±78.9 nanomole/g wet liver) within 6 hr and then a consecutive gradual increase until 48 hr (2004.9±170.4 nanomole/g wet liver) after the hepatectomy (Fig. 5).

The sham-operated group showed no significant changes in the hepatic content of hepatic polyamines. The time course of the polyamine contents in regenerating rat liver has a definite similarity with other studies (Moulinoux and Quemener, 1980; Zieve *et al.*, 1985; Luk, 1986).

In order to make sure of the isocratic HPLC method and the results described above, the influence of MGBG, an inhibitor of AdoMet dacarboxylase, on the polyamine contents in the regenerating liver was investigated.

Consequently, the change of both spermidine and spermine of the MGBG-treated group was not significantly different from those of the control group (Fig. 6). But the putrescine content of the MGBG-treated group, in contrast to that of the control group, showed a continuous increase to 1806.5±170.4 nanomole/g wet liver at 48 hr (Fig. 6), as previously reported (Fillingame and Morris, 1973; Morris, 1978). The present results, which are ratifing the previous studies (Pegg, 1983; Seppanen, 1983; Tabor and Tabor, 1984) on the inhibitory activity of MGBG on AdoMet-decarboxylase, are supporting the fidelity of the chromatographic data described in Table 1.

Therefore, it can be concluded that the isocratic reversed-phase HPLC method in combination with FNBT-derivatization described here is a

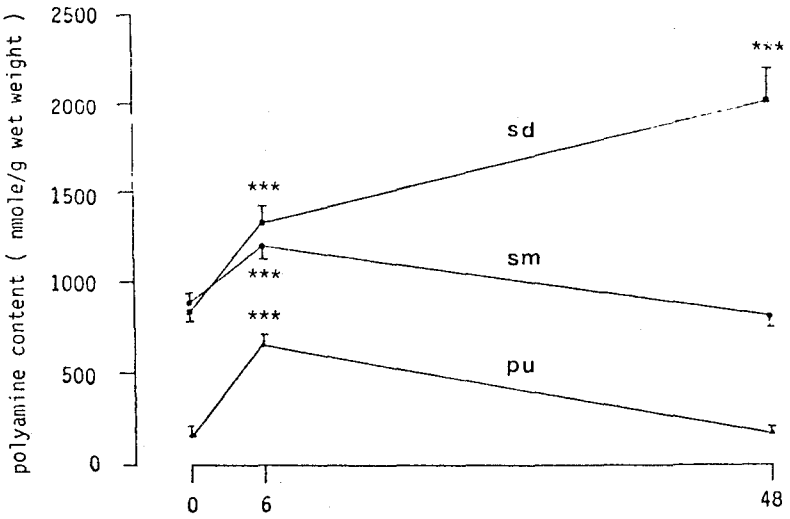

Fig. 5. Changes of polyamine content in the regenerating liver.
Data indicate the mean±standard error of 4 samples.
***indicate $p<0.005$.

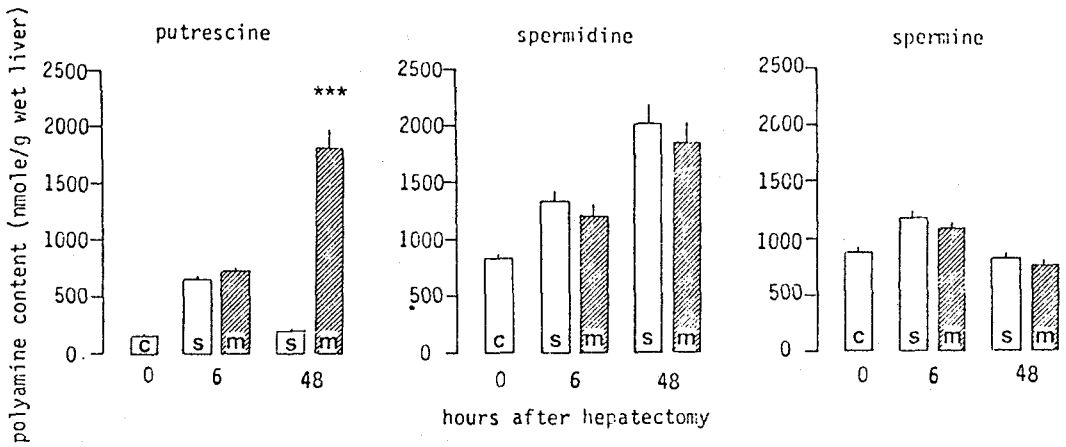

Fig. 6. Influence of MGBG on the change of polyamine content in the regenerating liver.
Abbreviations-c: control, s: isotonic saline, m: MGBG
Data indicate the mean±standard error of 4 samples.
***indicate $p<0.005$.

highly simple, specific, sensitive and reproducible method for the simultaneous and quantitative analysis of putrescine, spermidine and spermine of biological materials.

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═국문초록═

생체의 Polyamine-분석을 위하여 FNBT-유도체를 이용하는 간편하고 특이적이며 예민한 Isocratic RP-HPLC 분석법과 재생성 흰쥐-간의 Polyamine-대사의 변동에 관한 연구

고려대학교 의과대학 약리학교실

최상현 • 김형건 • 박홍익 • 전보권

복잡한 시료-전처리과정을 배제하여 간편하고 선택적이며 예민한 polyamine 측정방법으로써 4-fluoro-3-nitrobenzotrifluoride (FNBT) 유도체 및 단일이동상을 이용하는 역상의 고속액체 크로마토 그래피(HPLC) 방법과 아울러 이 방법을 이용한 흰쥐의 재생성간내 polyamine 대사의 변동에 관한 관찰-결과를 요약하면 다음과 같다.

1. 간내-polyamine은 0.4M perchloric acid에 추출한 후 Spragg와 Hutchings의 방법에 따라서 FNBT로 N-2'-nitro-4'-trifluoromethylphenyl polyamine (NTP-polyamine) 유도체를 만들고 이를 HPLC로 분석하였다.

2. HPLC-분석은 ERC ODS-1161 (3 um, 6×100 mm) column에서 분당 1.2 ml 속도의 단일-이동상(acetonitril 80%의 물)으로 분리하며 파장 242 nm로 검출-정량하였다.

3. HPLC-분석은 시료당 약20분히 소요되었는데, 크로마토그래피의 계치들에 있어서 각 polyamine의 capacity factor는 putrescine: 4.12, spermidine: 9.25, 및 spermine: 18.75로써 완전한 분리도를 보였으며, 검량한계는 10 picomole이하의 높은 예민도를 나타내었고, 시료처리-과정의 회수율은 약 72.1-88.5%로써 비교적 안정하였다.

4. Higgins와 Anderson의 방법에 따라서 흰쥐의 간을 부분-절제한 결과 70.4±1.99%가 절제되고 29.7±1.45%가 남게 되었으며, 수술후 48시간 후에 남은 간은 생리식염수-처치군 및 methylglyoxal bis (guanylhydrazone) dihydrochloride (MGBG)-처치군에서 각각 수술 전 간의 52.1% 및 53.3%로 회복되었다.

5. 정상 간의 polyamine 함량은 각각 putrescine: 158.7±14.1, spermidine: 829.7±36.4, 및 spermine: 875.6±42.1 nanomole/g wet liver이었고 ; 부분-절제하고 남은 간의 polyamine의 수술후 변동에 있어서, 생리식염수-처치군의 putrescine과 spermine은 6시간까지의 유의한 증가를 보인 다음 48시간에 정상치로 회복되었으나 spermidine은 48시간까지도 계속 증가하여 2004.9±170.4 nanomole/g wet liver가 되었다. MGBG-처치군의 spermidine 및 spermine 함량은 생리식염수군의 변동과 유사하였으나 putrescine의 함량은 6시간의 증가 후 계속 증가하여 48시간에는 1806.5±159.4 nanomole/g wet liver가 되었다.

이상의 성적으로 미루어 볼 때, 위의 단일 이동상의 역상-HPLC방법은 생체의 polyamine 정량분석을 위한 간편하고 예민하며 매우 선택적인 것으로 사료된다.